*IDENTIFICATION OF THE MEMBRANE PROTEIN AND "CORE" PROTEIN OF SINDBIS VIRUS**

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Electron micrographs of arbovirus (insect-borne animal viruses) virions suggest that they are composed of an outer lipoprotein envelope and an inner RNA-containing core that has also been assumed to contain proteins.[1, 2] The lipoprotein envelope of the virus is apparently acquired as the core buds through an altered cellular membrane.[2] Pfefferkorn and Hunter[3] analyzed purified preparations of Sindbis virus, a group A arbovirus, and found that 28 per cent of the mass of the virion was lipid. Because several analyses of animal cell membranes have yielded protein-to-lipid ratios of 1.6–2,[4, 5] Pfefferkorn[6] reasoned that the bulk of the total protein of the virion must be in the form of lipoprotein. When it was later found that all the viral protein was synthesized *de novo* after infection, the possibility was raised that the protein portion of the virus lipoprotein envelope was, in fact, specified by the virus genome. Because the RNA of this group of viruses is a molecule of about 2.0×10^6 daltons,[7] there was reasonable hope that their lipoprotein envelopes might be composed of only one polypeptide chain (or at most a few) combined with a specific set of lipids. The studies here reported indicate that the virion contains only two proteins—one associated with the viral RNA in a "core," the other associated with lipid to form the lipoprotein envelope of the virus.

Materials and Method.—Virus and tissue culture: Sindbis virus (HR strain)[8] was grown in monolayer cultures of fibroblasts prepared from 9- to 10-day-old chick embryos.[3] Cells were cultured in Eagle's medium[9] containing 5% calf serum.

Amino acid-labeled virus: Culture medium was removed and the cells were infected with Sindbis virus (50 PFU/cell delivered as 4 ml per 200-cm² surface area). After a 60-min adsorption period at 37°C, Eagle's medium supplemented with 3% rabbit serum and containing $^1/_{10}$ the normal amino acid concentration and 1 μc/ml of C^{14}-labeled "reconstituted algal protein hydrolysate" (Schwarz BioResearch Inc., Orangeburg, N.Y.) was added. Cell culture fluid was taken after 12 hr of incubation for virus yields. Virus-containing medium was layered over 15 ml of sucrose solution (15% sucrose, w/w, dissolved in buffer containing 0.05 *M* Tris, pH 7.5, 0.1 *M* NaCl, 0.3% rabbit serum) and sedimented in a Spinco SW25.2 centrifuge tube for 2 hr at 25,000 rpm, 4°C. The virus pellet was resuspended in the above buffer and subjected to zonal sedimentation through a linear sucrose gradient (15–30% w/w in 0.1 *M* NaCl, 0.05 *M* Tris pH 7.5, 0.3% rabbit serum). The presence of a small amount of serum in the sucrose solutions served both to preserve the infectivity of the virus and to prevent loss of virus during purification.

P^{32}-labeled virus: One day after primary chick fibroblast cultures were initiated, medium was removed and replaced by Eagle's medium with $^1/_{20}$ the normal phosphate concentration and 10 μc/ml carrier-free phosphoric acid P^{32} (Isoserve, Inc. Boston, Mass.). After 24 hr, P^{32} medium was removed, cells infected with Sindbis virus, and Eagle's medium with the normal phosphate concentration (unlabeled) added.

Virus was harvested 12 hr later and purified as described above. When cells are pre-

labeled in this way, 60–70% of the P^{32} of purified virus is present in phospholipid, with the remainder in viral RNA.[3]

Sedimentation data: Sedimentation coefficients of the virus and its subparticle were calculated by the method of Martin and Ames.[10] For this calculation the density of Sindbis virus was determined to be 1.19 by weighing an aliquot of sucrose-D_2O in which the virus banded. The density of the "core" particle was assumed to be between 1.3 and 1.5. Sedimentation coefficients of RNA were calculated relative to the 18*S* and 28*S* ribosomal RNA of HeLa cells.

Gel electrophoresis: After disruption of cells or virus with 0.1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol,[11] samples were dialyzed for at least 2 hr against 0.005 *M* sodium phosphate buffer, pH 7, 0.1% SDS, 0.1% 2-mercaptoethanol, and 10% sucrose. Subsequent procedures for electrophoresis, fractionation of gels, and radioactivity determinations have been described.[11]

Results.—Sindbis virus, labeled with a mixture of C^{14}- or H^3-amino acids, was disrupted by treatment with 1 per cent SDS and 1 per cent 2-mercaptoethanol. Simultaneous treatment with these reagents causes release (as individual molecules) of the constituent polypeptide chains of all complex proteins and viruses which have been tested (e.g., poliovirus,[11] adenovirus,[12] gamma globulin,[13] hemoglobin[14]). Electrophoresis of the denatured virus preparation was therefore expected to reveal the number of polypeptide chains in the virion. Only two such chains were detected (Fig. 1), which contain about 85 per cent of the radioactivity in the gel. Since the solutions employed in the electrophoresis contain SDS, all the protein-SDS complexes are negatively charged and migrate toward the anode and no protein can be lost in the system. The two chains found must therefore represent most, if not all, of the virion protein. (A polypeptide represented only

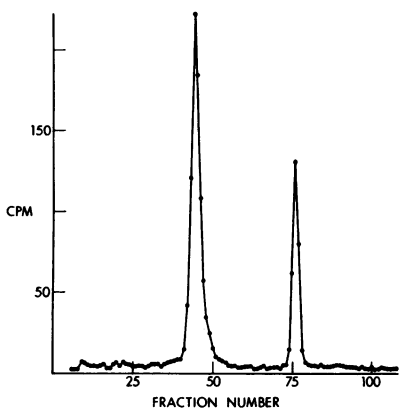

FIG. 1.—Polyacrylamide gel electrophoresis of Sindbis proteins. Purified Sindbis virus, labeled with C^{14}-amino acids, was treated with 1% SDS and 1% 2-mercaptoethanol and subjected to polyacrylamide gel electrophoresis. The gel was 10% acrylamide, 18 cm long. Electrophoresis was for 15 hr at 2.5 v/cm at room temperature. Migration is from left (*cathode*) to right (*anode*).

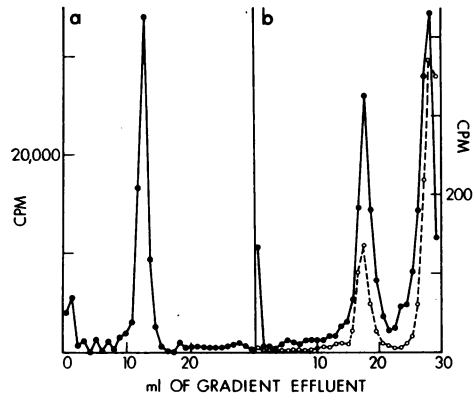

FIG. 2.—Sedimentation of Sindbis virus before and after DOC treatment. Sedimentation is from right to left through a 15–30% (w/w) sucrose gradient in 0.1 *M* NaCl, 0.05 *M* Tris pH 7.8, 0.3% horse serum, for 3 hr at 5°C at 25,000 rpm. The virus was labeled with C^{14}-amino acids (●) or with P^{32} (○). (Although not shown in part (*a*), P^{32}-labeled virus before treatment sedimented exactly the same as C^{14}-labeled virus.) (*a*) No treatment; (*b*) treated with 0.2% DOC.

once or twice per virion would not, however, have been detected.) The virus preparation used in the experiment shown in Figure 1 had been labeled with a mixture of 13 amino acids. The distribution of radioactivity between the two polypeptide chains should, then, approximate the mass distribution between the two proteins. On the average, 75 per cent of the radioactivity was found in the more slowly migrating peak.

To determine whether lipids would co-migrate with either protein peak, preparations of P^{32}-labeled virus were mixed with C^{14}-amino-acid-labeled virus and subjected to disruption and gel electrophoresis. None of the P^{32} co-migrated with the C^{14} protein, although 60–65 per cent of the P^{32} in such preparations was present in phospholipids (i.e., chloroform-methanol soluble). This was not surprising since SDS treatment might be expected to release the lipid from the protein. Other techniques tested, such as varying the detergent concentration or using instead 8 *M* urea treatment at various pH values, similarly failed to disrupt the virion in such a way that lipid and protein co-migrated in gel electrophoresis.

A different approach, based on the presumed morphology of the virion, was then tried. Since the membranous portion of the virus is thought to surround a nucleoprotein core,[1, 2] conditions were sought which would release the lipoprotein while leaving the core intact. Such a result was achieved by treatment of Sindbis virus with 0.2 per cent deoxycholate (DOC) (Fig. 2). Purified intact virus preparations, labeled with either C^{14}-amino acids or P^{32}, co-sedimented through a sucrose gradient with a sedimentation coefficient of 280*S*. After DOC treatment, labeled protein was found to sediment in two peaks, one at about 140*S*, the other at about 10–20*S*. Approximately 40 per cent of the P^{32} was associated with the 140*S* protein peak, and the remainder was released into material which remained at the top of the centrifuge tube (Fig. 2*b*). Thus it appears that this milder detergent treatment destroys the integrity of the virion but leaves intact some fairly large structure derived from the virion.

C^{14}-labeled protein from the two peaks of Figure 2*b* was examined by acrylamide gel electrophoresis in comparison with H^3-amino-acid-labeled virions. The results are shown in Figure 3. The protein from the 140*S* structure co-migrated with the faster-moving virus protein, while the DOC-released protein corresponded to the more slowly migrating protein.

The P^{32} in the 140*S* particle was found to be totally in RNA. It was insoluble in chloroform-methanol. Although acid-precipitable as isolated, it was rendered acid-soluble by treatment with 10 μg/ml of pancreatic RNase for 30 minutes at 37°C. Finally, treatment with SDS released RNA molecules which sedimented at about 35*S*, somewhat less than that observed for RNA isolated by SDS treatment of the intact virion.[15] This may reflect the RNase susceptibility of the 140*S* structure. Most (80%) of the P^{32} which had been virus-associated and released to the top of a sucrose gradient by DOC treatment was soluble in chloroform-methanol, and is therefore presumed to be in phospholipid.

Proteins synthesized in Sindbis-infected cells: The two proteins of mature Sindbis virus play an important role in the economy of Sindbis-infected chick embryo fibroblasts. As the infection proceeds, they form an increasing fraction of the total protein synthesized by the cell. When cells are infected in the pres-

ence of 1 μg/ml of actinomycin D and label is present from 7 to 10 hours after infection, more than 60 per cent of the incorporated radioactivity is found in the two virion proteins (Fig. 4). A more complete description of the proteins synthesized by the infected cell will be presented in a later publication.

Discussion.—The experiments presented here indicate that there are only two major proteins in the Sindbis virion. The protein that moves faster during

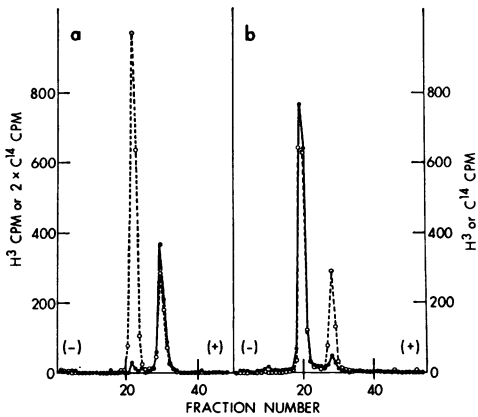

FIG. 3.—Polyacrylamide gel electrophoresis of Sindbis proteins isolated after DOC treatment. C^{14}-amino acid-labeled viral proteins (●) were isolated from the "core" region (*a*) of a sucrose gradient (see Fig. 2*b*), or from the top of the sucrose gradient (*b*), mixed with H^3-amino acid-labeled virus (O), and subjected to electrophoresis after treatment with 1% SDS–1% mercaptoethanol. The gel was 7.5% acrylamide, 10 cm long. Electrophoresis was for 16 hr at 0.8 v/cm at room temperature.

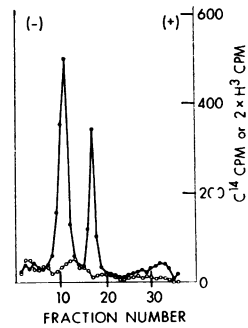

FIG. 4.—Electrophoresis of infected chick embryo fibroblast cytoplasm. Chick embryo fibroblasts were infected with Sindbis virus in the presence of 1 μg/ml of actinomycin D. After 7 hr of infection, the cell culture fluid was removed and replaced with medium containing C^{14}-amino acids and 1 μg/ml actinomycin D. Three hr later the culture fluid was again removed and the cell cytoplasm dissolved in 1% SDS. The labeled cytoplasm of these C^{14}-labeled infected cells (●) was mixed with H^3-labeled cytoplasm of uninfected cells (O) and subjected to electrophoresis (8 cm gel, 7.5%) as in Fig. 3. The two peaks of C^{14} protein seen in the figure have been shown in other experiments to co-migrate with the proteins of purified virus.

electrophoresis is present after detergent treatment of virus in a "core" particle that sediments at 140*S* and contains the virus RNA. The larger, more slowly migrating peptide is absent from the core particle and must therefore be the protein that serves as the structural protein in formation of the virus lipoprotein envelope. Since arboviruses acquire their membrane by budding through the altered plasma membrane of the infected cell,[2] it is probable that this protein alone, in conjunction with lipids, constitutes a functional biological membrane. Purified virus preparations could therefore yield pure membrane, a valuable experimental material. Since a large fraction of the protein being synthesized in infected chick fibroblasts corresponds to virus protein (Fig. 4), it should be possible to localize the membrane protein in cell fractions from the time of its synthesis until its entrance into membrane.

The potentialities of this particular system are greatly enhanced because of the genetic work already carried out with Sindbis virus. Burge and Pfefferkorn[8, 16] have isolated and characterized a number of conditional-lethal (temperature-sensitive) mutants of Sindbis virus. One class of these mutants may be defective in the virus-specified envelope or membrane protein: these mutants, when grown at a restrictive temperature, make infectious RNA and a particle similar if not identical to the core particle described in this paper, but do not make infectious virus.[17] When allowed to grow at permissive temperatures these mutants do produce infectious virus, but the particles are more heat-sensitive than wild-type particles, suggesting a defect in a structural component of the virion.

Sindbis virus thus appears to provide a potentially valuable experimental system for the genetic and biochemical investigation of a simple membrane.

Summary.—Purified preparations of Sindbis virus (a virus with a lipoprotein envelope) have been shown by gel electrophoresis to consist of two major proteins. Evidence is presented that one of these is a core protein associated with RNA, and the other serves as the protein portion of the lipoprotein envelope.

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